Insecticide Resistance Management

Navy Entomology Center of Excellence,
Jacksonville, FL
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FOREWORD

This pocket guide (PG) was written to consolidate information and procedures for managing insecticide resistance by the Navy Entomology Center of Excellence (NECE). This PG outlines methods to prevent, determine and react to insecticide resistance in insect disease vectors. It is not a regulation, but provides guidance to those individuals responsible for conducting pest control during military deployments. The guide will receive periodic review and will be updated to ensure that information presented reflects current technology and policy.

ACKNOWLEDGEMENTS

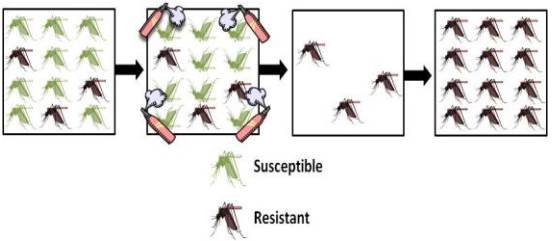
This NECE PG was written by LT James Harwood. Additional contributions were made by CDR Peter Obenauer, LCDR Craig Stoops, LCDR Carl Doud, Major Peter Nunn, LT Marcus McDonough, LT James Dunford, LT Hanayo Arimoto, Dr. Alec Richardson, Dr. Graham White of the USDA Center of Medical, Agricultural, Veterinary Entomology (CMAVE), and Dr. Robert Wirtz Chief of Entomology Branch at Centers for Disease Control and Prevention (CDC). Supportive information was acquired from the CDC, the World Health Organization (WHO), and the Insecticide Resistance Action Committee (IRAC).

CONTENTS

1. INTRODUCTION

WHAT IS PESTICIDE RESISTANCE?

Pesticide resistance is a decrease in a pest population's susceptibility to the mode of action of a pesticide, causing the pesticide to no longer control the pest population as efficiently. Pesticide resistance naturally occurs at low levels within a population, but the overuse of pesticides kills all the vulnerable individuals, leaving only the resistant pests alive. Resistance is then passed on to their offspring resulting in a higher percentage of resistant individuals in the population.



After a spray treatment, resistance is passed through the population from the survivors to the next generation

IMPLICATIONS

Pesticide resistance is not new or uncommon. It has been a side effect of insect vector control programs since 1914, and insect disease vectors in over 45 countries are resistant to at least one pesticide class. Consequently, there is a risk of pesticide resistance developing in any pest population anywhere.

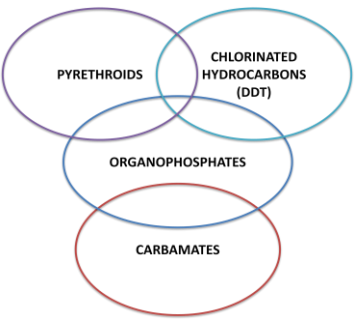
Pesticide resistance is a serious concern to military operations because it results in pests that are more difficult to control with conventional products, increased costs for management, and larger pest populations which can become a nuisance and pose serious health risks to personnel. Also, in nearly half of the recorded cases of insecticide resistance, there is resistance to two or more different classes of chemicals, due to the development of **multiple** and/or **cross resistance**. Pests that are resistant to many pesticides pose an especially difficult problem when chemical control is required.

MULTIPLE RESISTANCE

Multiple resistance occurs when two or more types of resistance independently develop within a population due to exposure to several different pesticides.

CROSS RESISTANCE

Cross resistance occurs when resistance to one pesticide results in resistance to other pesticides that have never been used against the pest. Cross resistances that occur among common insecticides are depicted below, with overlapping circles indicating which pesticide classes frequently share cross resistance.



TYPES OF RESISTANCE

There are two primary types of resistance:

1. **Physiological Resistance-** Most cases of pesticide resistance are due to a change in the pest population's physiology. Pests with physiological resistance can detoxify or destroy the pesticide toxins at a faster rate, break down the toxins inside the body, and/or prevent the pesticide from entering the body.

2. **Behavioral Resistance-** Pests that are behaviorally resistant to pesticides have changed their normal behavior, allowing them to avoid lethal doses of pesticides. This type of resistance is not as significant as physiological resistance in limiting a pest control program's success, but should be considered a potential contributing factor.

WHAT ARE THE FACTORS INFLUENCING RESISTANCE?

1. **Frequency of Pesticide Application:** The regularity of insecticide use directly affects the development of pesticide resistance in a population. Each time the insecticide is used, more of the susceptible individuals are killed, leaving behind an increasing proportion of resistant individuals to repopulate. Generally, the more often a single class of insecticide is used the more quickly resistance to it will develop.

2. **Dosage and Duration of Pesticide's Effect:** The amount of time that a pesticide is lethal to pests after its application (persistence) involves the product's formulation and application rate. Pesticides with a long persistence have the same effect as frequent applications on the development of resistance.

3. **Pest's Rate of Reproduction:** Pests with a short life cycles and high rates of reproduction are likely to develop resistance more quickly than pests that are longer lived with lower rates of reproduction.

4. **Isolation of the Pest Population:** The likelihood of resistance is also affected by the rate of immigration into the target population. If a population is being controlled close to one that is not, individuals from the uncontrolled area may migrate into the controlled population and restore some susceptibility. The opposite is true if there is little or no migration, since there will be no new susceptible individuals entering the population.

2. DETECTING RESISTANCE

The website http://www.pesticideresistance.org provides a list of all recorded instances of arthropod pesticide resistance since 1914, and is useful in determining the occurrence of resistance in specific geographic locations. However, absence of a record does not indicate a lack of resistance in a region, and a record of resistance does not suggest every population is resistant.

To determine if pesticide resistance occurs in a pest, resistance testing must be performed on individuals from the population. For mosquitoes, these assays include the Centers for Disease Control (CDC) bottle bioassay for adults and the World Health Organization's (WHO) susceptibility bioassay. A simplified procedure for each is provided here, but more detailed instruction can be accessed directly from the respective websites or by contacting a Navy Entomologist at NECE for support.

BIOASSAY FOR NON-MOSQUITO VECTORS/PESTS

While both the CDC and WHO bioassays can be performed on various insects, the remainder of the guide will focus specifically on how to detect resistance in mosquito vector populations. For a description of how to develop a bioassay for resistance testing in other groups of insects, refer to the following article and corresponding video:

Miller, A. L., Tindall, K., and Leonard, B. R ., 2010. Bioassays for Monitoring Insecticide Resistance (2010). ***J. Vis. Exp.*** (46), e2129

http://www.jove.com/video/2129/bioassays-for-monitoring-insecticide-resistance

BIOASSAY FOR MOSQUITO VECTORS

Before any bioassays can be conducted, wild mosquitoes must be sampled. For the CDC bottle assay, a minimum of 10 mosquitoes of each potentially resistant species are required for each pesticide tested, and a minimum of 10 are needed for the control. The WHO susceptibility bioassay requires 120-150 adult females of each species of interest per pesticide tested. The two recommended methods to acquire required samples of wild mosquitoes for resistance bioassays are:

1. **Collecting Wild Adults:** Use light or host-baited traps and other collecting methods, such as aspirators or resting boxes, to capture adults from the surrounding population. This will provide adults for testing immediately, but may result in small sample sizes and multiple species in each sample. If multiple species of mosquitoes are used, note whether they have blood-fed, if they are pre-gravid or gravid, and what species are being assayed.

2. **Field Collected Immatures:** Eggs, larvae, and pupae can be collected from breeding sites and reared to adults in the laboratory. While housing larval mosquitoes, it is important to provide a food source, consisting of a 3:2 mixture of bovine liver extract and brewer's yeast at a 2% solution in water. If the liver extract and yeast cannot be obtained, dry tropical fish food can be ground and provided to the larvae.

The adult field collected mosquitoes can be housed under laboratory conditions in group cages until the resistance testing begins. The adults will require cotton balls soaked in sugar water for food while being housed.

NOTE: The use of adults from laboratory laid eggs (F1 generation) is strongly discouraged when testing for resistance. This is due to the loss of genetic diversity that may occur in captive breeding populations, resulting in potentially more homozygous resistant individuals occurring in the laboratory population than in the natural population.

CDC BOTTLE BIOASSAY FOR ADULT MOSQUITOES

http://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html

The CDC bottle bioassay uses time mortality data that demonstrate the amount of time an insecticide takes to penetrate and act on the target sites of an insect. Any mechanisms that prevents or slows the compound from killing the insects, promotes resistance.

The CDC bottle bioassay can be used as part of a broader insecticide resistance monitoring program, which may include the WHO bioassay, and biochemical and molecular methods for identifying the mechanism of resistance.

MATERIALS

- Several same-sized clean empty bottles with lids (one bottle for each insecticide sample and one bottle to function as control)
- Acetone (reagent grade)
- Formulation grade insecticide
- Pipettes (one for each insecticide sample and one for adding acetone)
- Aspirator
- Digital timer capable of counting seconds
- Adult mosquitoes (10-20 for each bottle)

STEP 1: PREPARING THE FORMULATION

1. Add 1 ml of acetone to each bottle. Set one of these bottles aside to use as a control.
2. Add the insecticide to each of the assay bottles (the control only receives acetone). Be sure to use a separate pipette to avoid cross contamination of different insecticide samples (See table for suggested dosages).
3. Cap each bottle immediately to avoid premature evaporation of the insecticide.

Pesticide	µg / 250 ml bottle
Cyano-pyrethroids (deltamethrin)	25 µg/bottle
Permethrin	43 µg/bottle
Malathion	50 µg/bottle
Fenitrothion	40 µg/bottle
DDT	100 µg/bottle
Pyrethroids with synergists	30 µg/bottle

Diagnostic dosages for South American *Anopheles* spp. These should kill 100% of susceptible insects in 30 - 60 minutes. It is preferable to determine specific diagnostic doses for each region. Adapted from www.cdc.gov.

STEP 2: COATING THE BOTTLE

1. After adding the formulations to each bottle, coat the inside thoroughly by rotating the bottle in all directions. Be sure to also coat the inside of the lid during the process.
2. Remove the cap from each bottle, including the control bottle.
3. Roll each bottle on a table top until the acetone has evaporated. This usually takes less than 5 minutes.

STEP 3: LOADING MOSQUITOES

Note: The **first test** should be performed on mosquitoes known to be susceptible to calibrate the assay. Subsequent tests will involve mosquitoes from the populations that are thought to be resistant.

1. Transfer 10-20* test mosquitoes to each bottle using an aspirator.

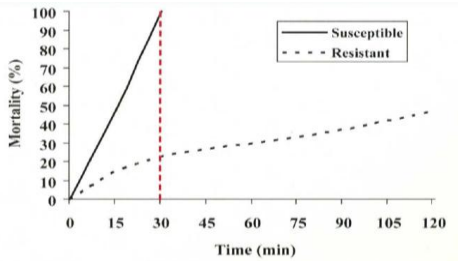
2. Puff **gently** to transfer the mosquitoes. Blowing too hard may cause the mosquitoes to hit the side of the bottle, killing them before the insecticide can.

*It is not required that each bottle have the same number of mosquitoes since you will be plotting the proportion of mosquitoes that die. However, it will be easier if the numbers in each bottle are approximately equal.

STEP 4: RECORDING MOSQUITO MORTALITY

The number of dead mosquitoes is recorded every **10 minutes,** with the first count conducted when the mosquitoes are placed in the bottle. Observations should be conducted until all mosquitoes have died or 2 hours have passed.

Graph the percentage of individuals that died every 10 minutes, as in the figure below. It helps to gently rotate the bottle while counting since dead mosquitoes slide with the curvature of the bottle. The CDC considers a mosquito to be dead if it can no longer stand.



www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf

STEP 5: INTERPRETING RESULTS

- The time required for all susceptible mosquitoes to die in the first test becomes the diagnostic time.
- If 98-100% of the potentially resistant mosquitoes die within the diagnostic time, there is no resistance to that pesticide.
- If 80-97% of the potentially resistant mosquitoes die within the diagnostic time, resistance may potentially exist, and further confirmation is needed.
- If less than 80% of the potentially resistant mosquitoes die within the diagnostic time, the population is most likely resistant to that pesticide.

STEP 6: CLEAN UP

- When you are finished with your bottles or they have become too old to use, triple rinse them with acetone and wash them with warm soapy water.
- Place the bottles in an oven to thoroughly dry before using them again.
- If you are uncertain whether the bottles are completely clean, introduce some susceptible mosquitoes into the beaker after drying them. The mosquitoes should not die right away. If they do, clean the beakers again.

WHO SUSCEPTIBILITY BIOASSAY FOR ADULT MOSQUITOES

http://www.who.int/malaria/publications/atoz/9789241505154/en/

The WHO susceptibility bioassay is a direct response-to-exposure test that involves a prefabricated test kit to measure mosquito mortality to a known standard dose of a given insecticide (i.e. the diagnostic or discriminating concentration).

Test kits include papers impregnated with insecticide at the appropriate diagnostic concentrations. The following procedure is a summary of the protocol. Detailed instructions will be provided with each test kit and can be found online at the link above.

AVAILABLE TEST KITS

As of December 2012, the following insecticide-impregnated test papers are routinely available for order:

Insecticide	Discriminating Concentration	Control Paper
Dieldrin	4% & 0.4%	Risella Oil
DDT	4%	Risella Oil
Malathion	5%	Olive Oil
Fenitrothion	1%	Olive Oil
Propoxur	0.1%	Olive Oil
Benfiocarb	0.1%	Olive Oil
Permethrin	0.75%	Silicone Oil
Deltamethrin	0.05%	Silicone Oil
Lambdacyhalothrin	0.05%	Silicone Oil
Cyfluthrin	0.15%	Silicone Oil
Etofenprox	0.5%	Silicone Oil

MATERIALS*

- 12 plastic tubes with each tube fitted at one end with 16-mesh gauze
- Six slide units, each fitted with a screw-cap on both sides and a 15 mm filling hole
- 40 sheets of clean paper (12 x 15 cm) for lining the holding tubes.
- 12 spring wire clips, 6 steel and 6 copper, to hold the paper in position against the walls of the tube
- Two glass or plastic aspirators
- One roll of self-adhesive plastic tape
- Instruction sheet and 20 copies of report forms
- 120-150 adult mosquitoes (20-25 per tube)

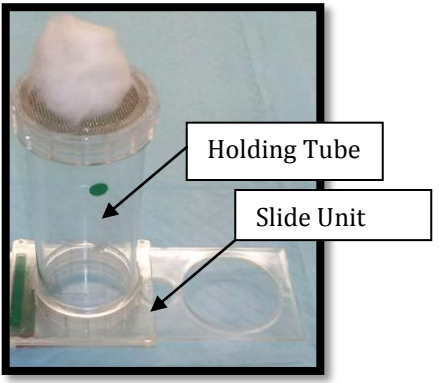
*Full kits are available from the World Health Organization for purchase by contacting:

Dr Zairi Jaal, Tel: 604-6574776; zairi@usm.my

http://www.who.int/whopes/resistance/en/WHO_CDS_CPE_PVC_2001.2.pdf

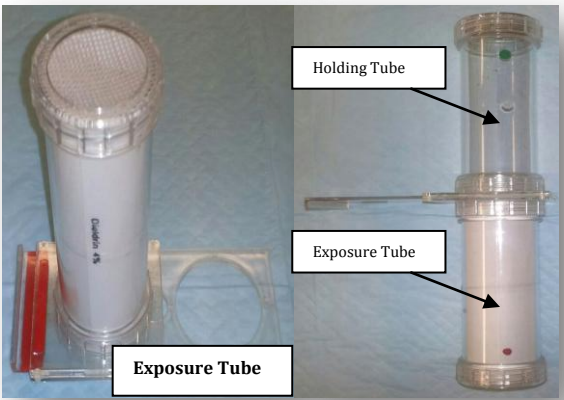
STEP 1: PREPARE THE HOLDING TUBE

1. Roll six sheets of clean white paper into a cylinder shape (12 x 15 cm).
2. Insert a rolled paper into each of six holding tubes (labeled green). Fasten the paper into position with a spring-wire clip. Place 20–25 mosquitoes into each holding tube using an aspirator.
3. Once the mosquitoes have been transferred, close the slide and place the holding tubes in an upright position for one hour, and then remove any damaged insects.



STEP 2: PREPARE EXPOSURE TUBES

1. Line the six exposure tubes (labeled red) with a sheet of insecticide-impregnated paper. Line the 2 control exposure tubes (labeled yellow) with oil-impregnated papers. Fasten all of the papers into position with a spring-wire clip.
2. Attach the exposure tubes to the opposite end of the holding tubes.



STEP 3: EXPOSE THE MOSQUITOES

1. Open the slide and gently blow the mosquitoes from the holding tubes into the exposure tubes. Once all the mosquitoes are in the exposure tubes, close the slide and remove the holding tubes.
2. Keep the mosquitoes in the exposure tubes, for 1 hour.
3. Transfer the mosquitoes back to the holding tubes after the 1-hour exposure period, by gently blowing on them. Then detach the exposure tube.
4. Place a cotton ball that has been soaked in sugar water on the mesh-screen end of the holding tubes to feed the adults during the recovery period.
5. Keep mosquitoes in the holding tubes for 24 hours (the recovery period).

STEP 4: COLLECT DATA

1. Record the number of dead mosquitoes after the 24 hour recovery period. Mosquitoes that are able to fly are considered alive. Any knocked-down mosquitoes are considered moribund and are counted as dead.
2. Transfer mosquitoes to individually labeled Eppendorf tubes (separating dead and live mosquitoes into separate tubes) for storage until they can be transferred to suitable facilities for species identification and supplementary testing if necessary.

NOTE: Pyrethroids and DDT are fast-acting insecticides with a knock-down effect. Observations should be made every 10 minutes for the first 1 hour when assessing these pesticides.

STEP 5: ANALYZE DATA

Calculate mortality by summing the number of dead mosquitoes across all four exposure replicates and determine the percent mortality as follows:

$$\frac{Number\ Dead}{Total\ Number\ Tested} X\ 100 = \%\ Mortality$$

Conduct a similar calculation for the control mortality. Discard the test if the control mortality is above 20%.

If the control mortality is between 5% and 20%, the observed mortality of the exposure tubes must be corrected using Abbot's formula, as follows:

$$\frac{\%\ Observed\ Mortality - \%\ Control\ Mortality}{100\% - Control\ Mortality} X\ 100$$

If the control mortality is below 5%, no correction is necessary.

STEP 6: INTERPRETING RESULTS

Data interpretation is based on the current WHO recommendations as follows:

- Mortality ranging from 98 to 100% indicates susceptibility.
- Mortality less than 98% suggests the presence of resistance, requiring further investigation.
- If mortality ranges from 90% to 97%, the presence of resistance in the population must be confirmed by performing additional bioassay tests with the same pesticide. If two additional tests consistently show mortality below 98%, resistance is confirmed.
- Mortality less than 90% confirms the existence of resistant genes in the population. Additional bioassays are not necessary, as long as at least 100 mosquitoes of **each** species were tested. Further investigation of the mechanisms and distribution of resistance should be conducted.

3. COMPARING CDC AND WHO TESTS

Both the CDC bottle assay and the WHO susceptibility test have specific advantages and disadvantages associated with their use. Depending on the situation, one test may be better suited than the other. The advantages and disadvantages of each test, as well as other comparisons to aid in selecting the best procedure to test for resistance, are presented in the following section.

The following comparisons are summarized from:

Aïzoun et al. 2013, Comparison of the standard WHO susceptibility tests and the CDC bottle bioassay for the determination of insecticide susceptibility in malaria vectors and their correlation with biochemical and molecular biology assays in Benin, West Africa, **Parasites & Vectors, 6:147**

Open access link:
http://www.parasitesandvectors.com/content/6/1/147

WHO SUSCEPTIBILITY TEST

	Advantages	Drawbacks
WHO	WHO papers are always ordered in the impregnated form	Requires careful transfer of mosquitoes from one tube to another
	Recording dead mosquitoes in WHO tubes is easy	Must monitor for 24 hours under controlled temperature and humidity
	Insecticide diagnostic doses recommended for susceptibility tests are standard	Cannot use synergists to detect metabolic resistance mechanisms
	WHO assay kits are purchased from a central source and allows easy comparison of results	Increasing cost of WHO kit and logistical complexity of the assay

CDC BOTTLE BIOASSAY

	Advantages	Drawbacks
CDC	CDC bioassay uses less mosquitoes	Bottles need to be coated with insecticide before each use
	Mosquitoes do not need to be transferred.	Shelf-life and re-use of pre-prepared bottles are still not documented
	Allows detection of simple or multiple resistance	Mortality recording in bottles is not easy
	Bottle assay is simple and rapid	
	Some of the components of bottle assay are more readily and cheaply available	
	Can test any concentration of any insecticide	
	Bottle bioassay can also test the efficacy of formulations	

4. MANAGING RESISTANCE

In order to delay or prevent the development of insecticide resistance in vector populations, resistance management must be considered in all vector control plans. Resistance management can still be conducted during operations using insecticide-based approaches.

- Before a vector control program begins, baseline data should be gathered on the populations' responses to the selected pesticides by conducting CDC bottle assays or WHO susceptibility test.

- During the vector control program, resistance should be monitored by conducting further bottle assays on a regular basis. Detection of resistance at an early stage allows timely management to be implemented.

METHODS TO REDUCE RESISTANCE

1. **Pesticide Rotation**
   Choose at least two different pesticides, each with a different mode of action (pesticide class), for the control program (see pg 39). Alternate between the different pesticides over time, usually using one for half of the control season and then switching to the other.

2. **Pesticide Mixtures**
   In this method, two or more pesticides are applied at the same time. This method assumes that if resistance to one pesticide is rare, then multiple resistance to all of the pesticides in a mixture will be extremely rare. The pest population must not be resistant to any of the pesticides in the mixture, and the pesticides must all be applied at their full application rate. Also, ensure that the product labels allow mixing of these pesticides and do not prohibit tank mixing.

3. Pesticide Mosaics

The "mosaic" approach involves applying different pesticides to different locations within an area. For example, spraying different pesticides into different dwellings within a village would be considered a mosaic approach. To maximize the efficacy of this method, adjacent areas should be treated with different pesticides, as in the figure below.

Example of a mosaic pesticide application. Each house is being treated by a pesticide with a different mode of action, as represented by the different colors. This same concept can be applied to outdoor areas, provided that neighboring sites are treated with different pesticides.

The modes of actions for six common pesticide groups used for controlling medically significant pest insects.

Primary Mode of Action	Group	Sub-group	Chemical Subgroup	Examples
Acetylcholinesterase (AChE) inhibitors	1	A	Carbamates	Bendiocarb, propoxur
		B	Organophosphates	Fenitrothion, malathion, temephos
Sodium channel modulators	3	A	Pyrethroids and pyrethrins	deltamethrin, permethrin, phenothrin
		B	DDT	DDT
Nicotinic acetylcholine receptor (nAChR) allosteric activators	5		Spinosyns	Spinosad
Juvenile hormone mimics	7	A	Juvenile hormone analogues	Methoprene, hydorpene
		C	Pyriproxyfen	Pyriproxyfen
Microbial disrupters of insect midgut membranes	11	A1	*Bacillus thuringiensis* var. *israelensis*	
		A2	*Bacillus sphaericus*	
Inhibitors of chitin biosynthesis type 0	15		Benzoylureas	Diflubenzuron, triflunuron, novaluron

GOOD WORK PRACTICES

As per DODINST 4150.07, all vector control programs should involve an integrated approach, so appropriate nonchemical methods should also be included in any insecticide resistance management plan.

Other good work practices that will help minimize the risk of resistance development include:

- Using pesticide at the label rate.
- Using pesticide applications less frequently.
- Using pesticides with short persistence.
- Avoid the same class of insecticide to control adults and juveniles.
- Leaving certain generations, population segments, or areas untreated.
- Establishing high pest densities or action thresholds prior to insecticide application.

5. REPONDING TO RESISTANCE

The appropriate course of action will depend on the circumstances. In some instances, modifying the resistance management strategy may help. The following actions should also be considered:

1. Use pesticides with caution and in conjunction with other nonchemical control measures.
2. Determine how widespread the resistance is in the area, if possible.
3. Contact NECE, the closest Navy Environmental and Preventative Medicine Unit (EPMU), U.S. Army Public Health Command (PHCR), or the Armed Forces Pest Management Board (AFPMB) for further assistance.

Resistance does not mean the pest population is uncontrollable, but control will require more effort and different management approaches. The medical entomologists at NECE can assist in planning any pesticide resistance management programs or provide support if resistance is suspected.

6. POINTS OF CONTACT

Contingency Liaison Officer
Armed Forces Pest Management Board
Email: afpmb-webmaster@osd.mil
COM: (301) 295-7476

Navy Entomology Center of Excellence
COM: (904) 542-2424
DSN: 942-2424

Navy Environmental Preventive Medicine Unit 2
COM: (757) 953-7671
DSN: 377-7671

Navy Environmental Preventive Medicine Unit 5
COM: (619) 556-7070
DSN 526-7070

Navy Environmental Preventive Medicine Unit 6
COM: (808) 473-0555
DSN: 473-0555

U.S. Army Public Health Command
PHCR-North Entomological Sciences Division
Email: PHCR-NorthESD@amedd.army.mil
COM: (301) 677-3466
DSN: (312) 622-3466

U.S. Navy and Marine Corps Public Health Center
COM: (757) 953-0700
DSN: 377-0700
www.nmcphc.med.navy.mil

7. REFERENCES

For more information the following references can be accessed online:

Arthropod Pesticide Resistance Database
http://www.pesticideresistance.org/

Centers of Disease Resistance Assays
http://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html

Insecticide Resistance Action Committee
http://www.irac-online.org/

World Health Organization Pesticide Evaluation Scheme (WHOPES)
http://www.who.int/whopes/resistance/en/

8. ACRONYMS

CDC	Centers for Disease Control and Prevention
DoD	Department of Defense
DODINST	Department of Defense Instruction
EPMU	Environmental Preventive Medicine Unit
IRAC	Insecticide Resistance Action Committee
NECE	Navy Entomology Center of Excellence
PG	Pocket Guide
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme